

Geniposide inhibits cell proliferation and migration in human oral squamous carcinoma cells via AMPK and JNK signaling pathways

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Abstract. Iridoids are a special class of cyclopentanoid monoterpenes, which exhibit a wide range of biological effects. The present study aimed to investigate the potential effects of three iridoids genipin, geniposide and geniposidic acid on three human oral squamous cell carcinoma (OSCC) cell lines HSC-2, SCC-9 and A253 in addition to studying the possible underlying mechanisms. Cell viability assay revealed that geniposide treatment significantly suppressed the proliferation of all three cancer cell lines. In addition, geniposide induced SCC-9 cell cycle arrest at the G₂/M phase (flow cytometry) through downregulation of cyclin-dependent kinase 2 and Cyclin A2 expression (western blot analysis), whilst also inducing cell apoptosis (flow cytometry and acridine orange/ethidium bromide staining) by dissipating the mitochondrial membrane potential (flow cytometry), and upregulating the expression of cleaved caspase-3 and

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cleaved poly-ADP ribose polymerase (western blot analysis). A wound-healing assay indicated that geniposide impaired SCC-9 cell migration by increasing the expression of E-cadherin (western blot analysis), whilst suppressing the expression of MMP-2 (western blot analysis). Western blot analysis also demonstrated that geniposide induced autophagy in SCC-9 cells by upregulating the expression of Beclin-1 and light chain 3-II. Mechanistically, geniposide activated the 5'-AMP-activated protein kinase signaling pathway and inhibited the JNK signaling pathway in SCC-9 cells (western blot analysis). The present results indicated that geniposide is able to inhibit the proliferation and migration of the tongue squamous carcinoma cell line SCC-9, suggesting a potential strategy for OSCC treatment.

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy worldwide and the most common oral malignancy, accounting for ~90% of cases (1). High-risk factors include periodontal diseases, viral infections, smoking and alcohol drinking habits, in addition to betel quid chewing (2,3). Despite developments in the treatment options over the past decade, such as surgery, chemotherapy and radiation, the overall survival rate remains poor (4). In particular, ~50% patients diagnosed with OSCC will typically succumb to this disease within 5 years (4). In addition, OSCC negatively impact the patients' quality of life by impairing taste and speech (5). Due to multidrug resistance, currently used chemotherapeutic strategies (such as 5-FU and cisplatin) for OSCC have low therapeutic efficacy and patients frequently suffer from unforeseen treatment failure in the clinical setting (6). Therefore, it remains in demand to develop novel anticancer methods for the treatment of OSCC.

Geniposide (Fig. 1) is a water-soluble iridoid glucoside that is mainly derived from the fruits of the flowering plant Gardenia jasminoides Ellis (Rubiaceae) and has been used as a traditional Chinese medicine for centuries (7). Several other sources of geniposide are known, including the small tree Eucommia ulmoides Oliv (8). In particular, hydrolysis products of geniposide, genipin is also found along with geniposide and several other derivatives like geniposidic acid (Fig. 1) (9). Various biological activities and pharmacological effects of geniposide, genipin and geniposidic acid have been previously reported, including anti-inflammatory, neuroprotective, antioxidative, anticancer, antidiabetic, hepatoprotective and cholagogic effects (7,10-14). In addition, previous studies have demonstrated that geniposide can significantly inhibit the proliferation of several cancer cell lines, such as diffuse large B-cell lymphoma cells (15), medulloblastoma cells (16) and gastric MKN45 cells (17). Lactobacillus and Lactobacillus casei strain Shirota have also been shown to enhance the antiproliferative effects of geniposide in the oral squamous carcinoma cell line HSC-3 (18,19). Furthermore, genipin has been reported to exert in vitro anticancer effects against colon cancer (20), bladder cancer (21), hepatocellular carcinoma (22), OSCC (23), colon (24) and gastric cancer cell lines (25,26). Geniposidic acid also has antitumor and radioprotective effects; Hsu et al (27) reported that 500 mg/kg of geniposidic acid significantly inhibited the growth of Erlich ascitic xenograft tumors in vivo, and preinadiation with geniposidic acid (500 mg/kg) showed positive effect on the recovery of leukocytes after 4 Gy sublethal irradiation.

The present study aimed to explore the effects of three iridoids on OSCC cell lines via CCK-8 assay, further investigate the roles of geniposide in apoptosis, cell cycle, cell migration and autophagy, as well as the underlying mechanisms via flow cytometry, AO/EB staining, wound healing assay and Western blot analysis. Finally, the regulation of geniposide on AMPK and JNK signaling was also studies. Taken together, the findings of the present study suggest that geniposide may be a promising lead compound for the development of clinical candidate for the OSCC treatment.

Materials and methods

Materials and chemical reagents. Genipin, geniposide and geniposidic acid were purchased from Dalian Meilun Biology Technology Co., Ltd. 5-Fluorouracil (5-FU) was purchased from MilliporeSigma. The chemical reagents were dissolved in DMSO to prepare stock solutions (100 μ M). Antibodies against cyclin-dependent kinase 2 (CDK2; cat. no. ab32147), cyclin A2 (cat. no. ab181591), cleaved caspase-3 (cat. no. ab32042), cleaved poly-ADP ribose polymerase (PARP; cat. no. ab32561), E-cadherin (cat. no. ab40772), MMP-2 (cat. no. ab92536), Beclin-1 (cat. no. ab210498), light chain 3 (LC3; cat. no. ab192890), 5'-AMP-activated protein kinase (AMPK; cat. no. ab207442), phosphorylated (p)-JNK1/2/3 (cat. no. ab124956) and JNK1/2/3 (cat. no. ab179461) were purchased from Abcam. Anti-p-AMPK (cat. no. 2535) antibody was obtained from Cell Signaling Technology, Inc. Anti-rabbit IgG (cat. no. KGAA35) and β -actin (cat. no. KGAA006) were purchased from Jiangsu KeyGen Biotech Co., Ltd. SP600125 (cat. no. T3109) and BML-275 (cat. no. T1977) was purchased from Shanghai Topscience Co., Ltd.

Cell culture. HSC-2, SCC-9 and A253 human OSCC cell lines were obtained from Cobioer Biosciences Co., Ltd. HSC-2 cells (cat. no. CBP60260) were cultured in minimum essential medium (MEM; cat. no. KGM41500-500; Jiangsu KeyGen Biotech Co., Ltd.) at 37°C with 5% CO₂. A253 cells (cat. no. CBP60662) were cultured in RPMI-1640 medium (cat. no. KGM31800-500; Jiangsu KeyGen Biotech Co., Ltd.) at 37°C with 5% CO₂. SCC-9 cells (cat. no. CBP60428) were grown in DMEM/F-12 (cat. no. KGM12500-500; Jiangsu KeyGen Biotech Co., Ltd.) supplemented with 10% heat-inactivated FBS (cat. no. KGY008; Jiangsu KeyGen Biotech Co., Ltd.) and 1% streptomycin/penicillin antibiotics at 37°C with 5% CO₂.

Cell viability. HSC-2, SCC-9 and A253 cells ($3.5x10^4$ cells/well) were seeded into 96-well plates and then incubated with 0.1% DMSO or 100 μ M test compounds (genipin, geniposide, geniposidic acid and 5-FU) at 37°C with 5% CO₂ for 72 h. Subsequently, 10 μ l CCK-8 solution (cat. no. KGA317; Jiangsu KeyGen Biotech Co., Ltd.) was added into each well before the cells were incubated for 2 h at 37°C. Following incubation, the absorbance of each well was recorded at 450 nm using a microplate reader (Elx800; BioTek Instruments, Inc.).

Dose-response of geniposide on SCC-9 cell viability. SCC-9 cells ($3.5x10^4$ cells/well) were seeded into 96-well plates and then incubated with 0.1% DMSO or geniposide (12.5, 25, 50 and $100 \,\mu$ M) at 37°C with 5% CO₂ for 48 h. Subsequently, $10 \,\mu$ l CCK-8 solution (cat. no. KGA317; Jiangsu KeyGen Biotech Co., Ltd.) was added into each well before the cells were incubated for 2 h at 37°C. Following incubation, the absorbance of each well was recorded at 450 nm using a microplate reader (Elx800; BioTek Instruments, Inc.).

Cell cycle analysis. SCC-9 cells $(3x10^5 \text{ cells/well})$ were seeded into six-well plates and incubated with 0.1% DMSO or geniposide (25, 50 and 100 μ M) at 37°C with 5% CO₂ for 48 h. The cells were then detached with trypsin and washed twice with PBS. Cells were then fixed with ice-cold 70% ethanol at 4°C overnight. Cells were then processed with a Cell Cycle Analysis Kit (cat. no. KGA511; Jiangsu KeyGen Biotech Co., Ltd.). The cells (5x10⁵) were incubated with 100 μ l RNase A at 37°C for 30 min and then with 400 μ l PI (both included in the kit) at 4°C for 30 min in the dark. The cell cycle distribution was then analyzed using a flow cytometer (BD CellQuest Pro version 6.0; BD Biosciences).

Apoptosis analysis. SCC-9 cells $(3x10^5 \text{ cells/well})$ were seeded in six-well plates and incubated with 0.1% DMSO or 25, 50 and 100 μ M geniposide at 37°C with 5% CO₂ for 48 h. The cells were then trypsinized, washed twice with PBS and collected by centrifugation at 500 x g for 5 min at 20°C. Cells (5x10⁵) were re-suspended in 500 μ l binding buffer (cat. no. KGF005; Jiangsu KeyGen Biotech Co., Ltd.) and stained with 5 μ l Annexin V-APC and 5 μ l 7-AAD (cat. no. KGA1024; Annexin Apc V 7 AAD Apoptosis Detection Kit; Jiangsu KeyGen Biotech Co., Ltd.) for 15 min in the dark. Subsequently, cells were analyzed using a flow cytometry (BD FACSCaliburTM; BD Biosciences) and data were analyzed with BD CellQuest Pro version 6.0 (BD Biosciences). Dot-plots were assessed for the





Figure 1. Structures of geniposide and its analogues.

percentage of cells considered to be live (lower left quadrant), early apoptotic (lower right quadrant), late apoptotic (upper right quadrant), and necrotic (upper left quadrant). Apoptotic index=apoptotic cell number/total cell number x100%.

Mitochondrial membrane potential ($\Delta \Psi m$) analysis. SCC-9 cells (3x10⁵ cells/well) were seeded into six-well plates and incubated with 0.1% DMSO or 25, 50 and 100 µM geniposide at 37°C with 5% CO₂ for 48 h. The cells were then trypsinized, washed with PBS and collected by centrifugation at 800 x g for 5 min at 20°C. Cells (1x10⁶) were re-suspended in 500 µl incubation buffer containing tetramethylrhodamine methyl ester staining (JC-1; cat. no. KGA602; Jiangsu KeyGen Biotech Co., Ltd.) and incubated at 37°C for 15 min in a 5% CO_2 incubator. The cells were collected by centrifugation at 500 x g for 5 min at room temperature, re-suspended and then analyzed using flow cytometry (BD CellQuest Pro version 6.0; BD Biosciences). The relative $\Delta \Psi m$ was expressed as the ratio of JC-1 red fluorescence of normal mitochondrion detected in the red channel (FL2-H) (upper right quadrant) to green fluorescence of low-potential mitochondria detected in the green FITC channel (FL1-H) (lower right quadrant).

Acridine orange/ethidium bromide (AO/EB) staining. SCC-9 cells ($1x10^5$ cells/well) were seeded into six-well plates and incubated with 0.1% DMSO or 25, 50 and 100 μ M geniposide at 37°C with 5% CO₂ for 48 h. The cells were then stained with 100 μ l AO/EB dyemix (cat. no. KGA213; Jiangsu KeyGen Biotech Co., Ltd.) at room temperature for 15 min in the dark and then washed twice with PBS. The apoptotic cells were visualized under a fluorescence microscope (IX51; Olympus Corporation) at x100 magnification.

Wound-healing assay. SCC-9 cells (1x10⁶ cells/well) were seeded into six-well plates and grown to 80% confluence at 37°C in serum-free culture medium (DMEM/F12). The cells were then scratched using a tip (1 ml) and washed with PBS. These SCC-9 cells were incubated with 0.1% DMSO or 25, 50 and 100 μ M geniposide at 37°C with 5% CO₂ in serum-free culture medium (DMEM/F12) for 48 h. Images were captured at 0 and 48 h timepoints with a phase contrast light microscope (magnification, x100; IX51; Olympus Corporation) and the migration distance in each group was determined.

Western blot analysis. After culturing, SCC-9 cells were treated with 0.1% DMSO or 25, 50 and 100 μ M geniposide at 37°C with 5% CO₂ for 48 h. For p-JNK1/2/3 or JNK1/2/3 or p-AMPK or AMPK, SCC-9 cells were pre-treated for 1 h

with/without 10 µM SP600125 or 10 µM BML-275 before exposure to 100 µM geniposide at 37°C with 5% CO₂ for 48 h. The cells were then collected by trypsinization, washed with PBS and then lysed with ice-cold cell lysis buffer for western and immunoprecipitation (cat. no. KGP701; JiangsuNanjing KeyGen Biotech Co., Ltd). After centrifugation at 24,080 x g for 15 min at 4°C, the protein samples were collected and quantified using the Bradford assay. Equal amounts of proteins (30 µg/lane) were separated by 10% SDS-PAGE, before the separated proteins were transferred onto nitrocellulose membranes (cat. no. 66485; Pall Corporation). After blocking in 5% non-fat milk (Anchor Corporation) at room temperature for 2 h, the membranes were washed three times with tris-buffered saline containing Tween-20 (TBST; cat. no. KGP109-T; Jiangsu KeyGen Biotech Corp., Ltd.) and incubated overnight at 4°C with primary antibodies against CDK2 (1:2,000 dilution), cyclin A2 (1:2,000), cleaved caspase-3 (1:500), cleaved PARP (1:1,000), E-cadherin (1:10,000), MMP-2 (1:2,000), Beclin-1 (1:1,000), LC3 (1:2,000), AMPK (1:1,000), p-JNK1/2/3 (1:2,000), JNK1/2/3 (1:1,000), p-AMPK (1:1,000) and β -actin (1:1,000). Following primary antibody incubation, membranes were washed three times with TBST and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:4,000) for 1 h at room temperature. Immunolabeling was then visualized using an enhanced chemiluminescence kit (cat. no. KGP116; Jiangsu KeyGen Biotech Co., Ltd.). Densitometric analysis of the protein bands was performed with Gel-Pro Analyzer version 4.0 software (Media Cybernetics Inc.).

Statistical analysis. Data are presented as the mean \pm standard deviation of three independent experiments. Data were analyzed using GraphPad Prism 8.3.0 (GraphPad Software, Inc.). Multiple comparisons were performed using one-way ANOVA test with Geisser-Greenhouse correction and Dunnett's post-hoc test. Comparison of each group vs. every other group was performed by one-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxic effects of genipin, geniposide and geniposidic acid on OSCC cells. The HSC-2, SCC-9 and A253 human OSCC cell lines were incubated with genipin, geniposide and geniposidic acid (all 100 μ M) for 72 h before cell viability was measured using CCK-8 assay. Genipin and geniposidic acid slightly inhibited the viability of HSC-2, SCC-9 and A253 cells (Fig. 2). Geniposide had the strongest antiproliferative activity in all three OSCC lines (Fig. 2). Treatment with geniposide significantly reduced cell viability by >50% in HSC-2, SCC-9 cells and A253 cells, the extent of which was comparable with that mediated by the clinical drug 5-FU in all three cell lines (Fig. 2).

Geniposide suppresses the viability of SCC-9 cells in a concentration-dependent manner. The possible effects of different concentrations of geniposide (12.5, 25, 50 and 100 μ M) on the viability of tongue squamous cell carcinoma cell line SCC-9 were investigated further using CCK-8 assay.



Figure 2. Effects of genipin, geniposide and geniposidic acid treatments on cell viability. Results of Cell Counting Kit -8 assay performed on (A) HSC-2, (B) SCC-9 and (C) A253 cells following incubation with $100 \,\mu$ M of the respective compounds for 72 h. Data are presented as the mean ± standard deviation. Experiments were performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs. Control group. Multiple comparisons were performed using one-way ANOVA with Geisser-Greenhouse correction and Dunnett's post-hoc test.



Figure 3. Dose-response of geniposide on SCC-9 cell viability. Results of the Cell Counting Kit-8 assay following incubation with 12.5, 25, 50 and 100 μ M geniposide for 48 h. Data are presented as the mean ± standard deviation. Experiments were performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs. Control group. Multiple comparisons were performed using one-way ANOVA with Geisser-Greenhouse correction and Dunnett's post-hoc test.

Geniposide treatment significantly suppressed the viability of SCC-9 cells in a concentration-dependent manner (Fig. 3).

Geniposide induces G_2/M arrest and downregulates CDK2 and cyclin A2 expression in SCC-9 cells. To determine if the inhibitory effects of geniposide on SCC-9 cell viability resulted from cell cycle arrest, the effects of geniposide on cell cycle distribution was investigated using flow cytometry after labeling with PI. The cells were therefore incubated with 25, 50 and 100 μ M geniposide for 48 h. Treatment with geniposide induced cell cycle arrest at G_2/M phases and significantly reduced the number of cells in the G_0/G_1 phase in a dose-dependent manner, compared with those in the control group (Fig. 4A and B). In addition, the effect of geniposide on the expression levels of cell cycle regulators in SCC-9 cells was also evaluated. Cells were incubated with 25, 50 and 100 μ M geniposide for 48 h, before the expression levels of CDK2 and Cyclin A2 were measured through western blot analysis. Geniposide resulted in the downregulation of both CDK2 and CyclinA2 in a dose-dependent manner, which may have been the underlying reason for the geniposide-induced G₂/M phase arrest observed (Fig. 4C and D).

Geniposide induces apoptosis by decreasing the mitochondrial membrane potential and increasing the expression levels of cleaved caspase-3 and cleaved PARP in SCC-9 cells. To study the effects of geniposide on SCC-9 cell apoptosis, flow cytometry and western blot analysis were performed. Cells were incubated with 25, 50 and 100 μ M geniposide for 48 h, before the percentages of apoptotic cells were analyzed using flow cytometry after labeling with Annexin V-FITC/7-AAD. Geniposide promoted the apoptotic ratio of SCC-9 cells in a dose-dependent manner. After treatment with 25, 50 and 100 μ M geniposide, the percentages of apoptotic cells were all significantly greater compared with those in the control group (Fig. 5A and B). Following incubation with 25, 50 and 100 μ M geniposide for 48 h and further staining with JC-1, the mitochondrial membrane potential in SCC-9 cells was also significantly decreased from 12.56 to 38.37% (Fig. 5C and D). In addition, geniposide significantly increased the expression levels of cleaved caspase-3 and cleaved PARP in a dose-dependent manner in SCC-9 cells (Fig. 5E and F). These results suggest that geniposide can induce SCC-9 cell apoptosis by reducing the mitochondrial membrane potential, in addition to upregulating the expression of cleaved caspase-3 and cleaved PARP.

AO/EB staining observation of apoptosis. Subsequently, the effects of geniposide on the apoptosis of SCC-9 cells was visualized using AO/EB staining. Cells exhibited normal morphology with uniform green staining in the control group (Fig. 6). However, light orange fluorescence, chromatin condensation and shrinkage were observed in SCC-9 cells





Figure 4. Geniposide induces cell cycle arrest in SCC-9 cells. (A) Flow cytometry analysis of cell cycle distribution following 48 h incubation with 25, 50 and 100 μ M geniposide. (B) Quantitative analysis of cell cycle distribution. (C) Western blot analysis of CDK2 and cyclin A2 expression levels in SCC-9 cells following 48 h incubation with 25, 50 and 100 μ M geniposide. (D) Western blotting quantification. Data are presented as the mean ± standard deviation. Experiments were performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs. Control group. Multiple comparisons were performed using one-way ANOVA with Geisser-Greenhouse correction and Dunnett's post-hoc test.



Figure 5. Effects of geniposide on SCC-9 cell apoptosis. (A) Flow cytometry analysis of apoptotic SCC-9 cells stained with Annexin-V-FITC/7-AAD following 48 h incubation with 25, 50 and 100 μ M geniposide. (B) Quantification of apoptotic SCC-9 cells. (C) Flow cytometry analysis of the mitochondrial membrane potential in JC-1-stained SCC-9 cells following 48 h incubation with 25, 50 and 100 μ M geniposide. (D) Quantitative analysis of the expression of cleaved caspase-3 and cleaved PARP in SCC-9 cells following 48 h incubation with 25, 50 and 100 μ M geniposide. (F) Western blotting quantification. Data are presented as mean ± standard deviation. Experiments were performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs. Control group. PARP, poly-ADP ribose polymerase. Multiple comparisons were performed using one-way ANOVA with Geisser-Greenhouse correction and Dunnett's post-hoc test.



Figure 6. Effects of geniposide on AO/EB staining in SCC-9 cells. The cells were incubated with geniposide (25, 50 and 100 μ M) for 48 h, before being stained with AO/EB. Live cells in green and apoptotic cells in red were visualized using fluorescence microscopy. Scale bars, 50 μ m. AO, acridine orange; EB, ethidium bromide.

incubated with geniposide, suggesting that geniposide can induce SCC-9 cell apoptosis (Fig. 6).

Geniposide inhibits the migration of SCC-9 cells. The effects of geniposide on SCC-9 cell migration was next evaluated using wound healing assay. Geniposide treatment significantly decreased the migration of SCC-9 cells after 48 h in a dose-dependent manner compared with that in the Control group (Fig. 7A and B). Furthermore, geniposide significantly increased the expression of E-cadherin, whilst significantly suppressing the expression of MMP-2 in SCC-9 cells in a dose-dependent manner compared with those in the Control group (Fig. 7C and D).

Effect of geniposide on the expression autophagy markers in SCC-9 cells. Since autophagy is a crucial programmed cell death process (28), it was next investigated whether autophagy was involved in geniposide-reduced SCC-9 cell death. The effect of geniposide on the expression levels of autophagy markers Beclin1 and LC3 in SCC-9 cells was evaluated. Treatment with geniposide significantly increased the expression levels of Beclin1 and LC3, in particular increasing the generation of LC3-II, compared with those in the Control group (Fig. 8). These findings suggest that geniposide treatment enhanced SCC-9 cell autophagy. Effect of geniposide on AMPK and JNK signaling in SCC-9 cells. A previous study reported that the AMPK and JNK signaling pathways are key regulators of apoptosis and autophagy (29). To assess the effect of geniposide on the AMPK and JNK pathways in SCC-9 cells, western blot analysis was performed to analyze the phosphorylation levels of AMPK and JNK. Compared with those in the control group, the levels of AMPK phosphorylation and expression were increased in the geniposide group (Fig. 9A and B). However, in Fig. 9C, geniposide was not observed to affect the p-AMPK/AMPK ratio compared with that in the control group. In addition, 10 μ M AMPK inhibitor BML-275 significantly reversed the upregulation of AMPK phosphorylation and AMPK expression caused by geniposide treatment. Decreased JNK phosphorylation and JNK expression were observed in the geniposide group (Fig. 9D-E). By contrast, the downregulation in the levels of JNK phosphorylation and JNK expression originally caused by geniposide were significantly potentiated after pre-treatment with 10 µM JNK inhibitor SP600125, compared with those in the control group (Fig. 9D-E). While, in Fig. 9F, geniposide was not observed to affect the p-JNK/JNK ratio, compared with the control group. These data suggest that geniposide activated the AMPK signaling pathway but inhibited the JNK signaling pathway in SCC-9 cells.





Figure 7. Geniposide inhibits SCC-9 cell migration. (A) Wound healing assay of SCC-9 cells incubated with 25, 50 and 100 μ M geniposide for 48 h. Images were acquired at 0 and 48 h. Scale bars, 100 μ m. (B) Migration length. (C) Western blot analysis of E-cadherin and MMP-2 expression in SCC-9 cells following incubation for 48 h with 25, 50 and 100 μ M geniposide. (D) Western blotting quantification. Data are presented as the means ± standard deviation. Experiments were performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs. Control group. Multiple comparisons were performed using one-way ANOVA test with Geisser-Greenhouse correction and Dunnett's post hoc test.



Figure 8. Geniposide upregulates SCC-9 cell autophagy. (A) Effects of geniposide on the expression levels of Beclin-1 and LC3 in SCC-9 cells. The cells were incubated with geniposide (25, 50 and 100 μ M) for 48 h, before the protein expression levels were detected using western blot analysis. (B) Western blotting quantification. Data are presented as the mean ± standard deviation. Experiments were performed in triplicate. *P<0.05 and **P<0.01 and vs. Control group. Multiple comparisons were performed using one-way ANOVA with Geisser-Greenhouse correction and Dunnett's post-hoc test.

Discussion

Geniposide is a major active ingredient in the fruit of *Gardenia jasminoides* Ellis (9). It has been previously demonstrated that geniposide has various biological properties, including anti-inflammatory, neuroprotective, anticancer, antidiabetic and cholagogic effects (9). Previous studies showed that geniposide can exert significant cytotoxicity towards several cancer cell lines, such as diffuse large B-cell lymphoma cells, medulloblastoma cells and gastric MKN45 cells (15-17). In addition, Cheng *et al* (18) demonstrated that *Lactobacillus* can improve the *in vitro* antineoplastic effects of geniposide on the human OSCC cell line HSC-3 through β -glucosidase production to transform geniposide into genipin. Similarly, Qian *et al* (19) discovered that treatment with *Lactobacillus casei* strain Shirota enhanced the *in vitro* anti-proliferative effects of

geniposide on HSC-3 cells. In the present study, geniposide significantly decreased cell viability in three OSCC cell lines tested. In addition, geniposide suppressed the viability of HSC-2, SCC-9 and A253 cells by levels comparable to those mediated by the clinic drug 5-FU. In particular, geniposide significantly inhibited the viability of SCC-9 cells in a concentration-dependent manner.

Cell cycle regulators CDK2 and cyclin A2 serve important roles in regulating the cell cycle transitions, specifically at progression through the S and G₂ cell cycle phases (30). Hwang *et al* (31) previously demonstrated that geniposide can increase DU145 cell accumulation at the sub-G₁ phase. The present study revealed that geniposide induced G₂/M phase arrest in SCC-9 cells by downregulating the expression of CDK2 and cyclin A2.

Caspase-3 and PARP are involved in the regulation of cell apoptosis, such that the presence of cleaved caspase-3 and



Figure 9. Effects of geniposide treatment on the AMPK and JNK signaling pathways in SCC-9 cells. SCC-9 cells were pre-treated for 1 h with/without $10 \,\mu$ M BML-275 or $10 \,\mu$ M SP600125 before exposure to $100 \,\mu$ M geniposide for 48 h. Western blot analysis of the (A) phosphorylation and expression of AMPK, (B) which were quantified. (C) p-AMPK/AMPK ratio was calculated. Western blot analysis of the (D) phosphorylation and expression of JNK1/2/3, (E) which were quantified. (F) The ratio of p-JNK1/2/3 and JNK1/2/3 was calculated. Relative protein expression levels were normalized to those of the β -actin loading control. Data are presented as the mean \pm standard deviation. Experiments were performed in triplicate. *P<0.05 and **P<0.01 and vs. Control group. *P<0.05, #*P<0.01 and ###P<0.001. AMPK, 5'-AMP-activated protein kinase. Multiple comparisons were performed using one-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple-comparison test.

cleaved PARP are considered to be markers of apoptosis (32). In the present study, flow cytometry and western blot analyses indicated that geniposide significantly increased the apoptotic ratio in SCC-9 cells by increasing the expression levels of cleaved caspase-3 and cleaved PARP. Recently, Chen *et al* (16) reported that geniposide treatment resulted in *in vitro* anticancer activity in Daoy medulloblastoma cells by upregulating the expression level of cleaved caspase 3.

Tumor metastasis is one of the main causes of chemotherapy failure and neoplasm recurrence (33). Cell surface E-cadherin and MMP-2 are proteins associated with cancer cell migration and tumor metastasis (34). In the present study, wound healing assay results showed that geniposide decreased SCC-9 cell migration, possibly by increasing E-cadherin expression whilst decreasing MMP-2 expression. It has been previously reported that treatment with geniposide inhibited Daoy cell migration by suppressing MMP-2 expression (16).

Autophagy serves a key role in maintaining cell homeostasis and survival under stressful conditions and has a significant effect on health (cell aging and differentiation) and disease (liver cirrhosis, myocardial infarction and heart failure) (35). However, the role of autophagy in cancer is dichotomous, since it has been found to both inhibit tumor initiation and promote tumor progression (36). In the present study, western blot analysis indicated that geniposide activated SCC-9 cell autophagy by upregulating the expression of Beclin-1 and LC3-II. To the best of our knowledge, the present study was the first to report that the *in vitro* anticancer effects of geniposide is associated with the regulation of autophagy.

AMPK is a key sensor of cellular energy homeostasis in mammalian cells and serve an important regulatory role in the metabolism of carbohydrates and fats (37). By contrast, JNK is a member of the MAPK family that has been reported to regulate cell proliferation and differentiation (38). AMPK and JNK have been previously shown to regulate apoptosis and autophagy (39). In the present study, it was observed that geniposide could stimulate AMPK signaling pathway whilst inhibiting the JNK signaling pathway in SCC-9 cells.

In conclusion, results of the present study indicated that geniposide can be an effective *in vitro* antineoplastic agent against OSCC cells by acting through multiple mechanisms. Potential mechanisms include cell cycle arrest at the G_2/M phase through



downregulation of CDK2 and cyclin A2 expression, cell apoptosis by disturbing the mitochondrial membrane potential and upregulating cleaved caspase-3 and cleaved PARP expression, inhibition of cell migration by increasing the E-cadherin/MMP-2 expression ratio, cell autophagy through upregulation of Beclin-1 and LC3-II expression, in addition to the regulation of AMPK and JNK signaling. Although geniposide exhibited inhibitory effects against three OSCC cell lines similarly to the clinical drug 5-FU, further investigations into the *in vivo* effectiveness and efficiency of geniposide should be conducted, with focus on the toxicity and pharmacokinetics of this agent.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GB, BC, XX, YL, XL and DanZ performed the experiments, collected data and wrote the manuscript. GB, LZ and DegZ analyzed the data and wrote the manuscript. GB and LZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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